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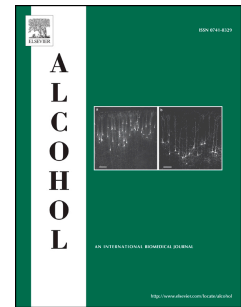
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**Early gestational ethanol exposure in mice: effects on brain structure, energy metabolism and adiposity in adult offspring**

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Declarations of interest: none

**Abstract**

We examined whether an early-life event — ethanol exposure in the initial stages of pregnancy — affected offspring brain structure, energy metabolism and body composition in later life. Consumption of 10% (v/v) ethanol by inbred C57BL/6J female mice from 0.5 to 8.5 days post coitum was used to model alcohol exposure during the first 3-4 weeks of gestation in humans, when pregnancy is not typically recognized. At adolescence (postnatal day (P) 28) and adulthood (P64), the brains of male offspring were scanned *ex vivo* using ultra-high field (16.4 Tesla) magnetic resonance imaging and diffusion tensor imaging. Energy metabolism and body composition were measured in adulthood by indirect calorimetry and dual-energy X-ray absorptiometry (DXA), respectively. Ethanol exposure had no substantial impact on white matter organization in the anterior commissure, corpus callosum, hippocampal commissure, internal capsule, optic tract or thalamus. Whole brain volume and the volumes of the neocortex, cerebellum and caudate putamen were also unaffected. Subtle, but non-significant, effects were observed on the hippocampus and the hypothalamus in adult ethanol-exposed male offspring. Ethanol exposure was additionally associated with a trend towards decreased oxygen consumption, carbon dioxide production and reduced daily energy expenditure as well as significantly increased adiposity, albeit with normal body weight and food intake, in adult male offspring. In summary, ethanol exposure restricted to early gestation had subtle long-term effects on the structure of specific brain regions in male offspring. The sensitivity of the hippocampus to ethanol-induced damage is reminiscent of that reported by other studies — despite differences in the level, timing and duration of exposure — and likely contributes to the cognitive impairment which characteristically results from prenatal ethanol exposure. The hypothalamus plays an important role in regulating metabolism and energy homeostasis. Our finding of altered daily energy expenditure and adiposity in adult ethanol-exposed males is consistent the idea that central

nervous system abnormalities also underpin some of the metabolic phenotypes associated with ethanol exposure in pregnancy.

## **Keywords**

prenatal, alcohol, brain structure, energy metabolism, body composition

## **Introduction**

Adverse early-life environmental exposures such as gestational undernutrition, overnutrition and diabetes mellitus have been shown to increase susceptibility to obesity and its related diseases in later life (Curhan et al., 1996; Fraser et al., 2010; Law, Barker, Osmond, Fall, & Simmonds, 1992; Lunde et al., 2016; Ravelli, van Der Meulen, Osmond, Barker, & Bleker, 1999; Zhao et al., 2016). The underlying mechanisms are not fully understood; however, affected pathways include those involved in the control of appetite (Bellinger, Lilley, & Langley-Evans, 2004; Franke et al., 2005), glucose metabolism (Phillips, Barker, Hales, Hirst, & Osmond, 1994), circadian rhythms (Borengasser et al., 2014; Sutton, Centanni, & Butler, 2010) and adipogenesis (Borengasser et al., 2013; Lukaszewski et al., 2011).

There is growing evidence that prenatal ethanol exposure has similar consequences, with links to increased adiposity, insulin resistance and glucose intolerance in animal models (Chen & Nyomba, 2003; Dobson et al., 2012; Gardebjer, Anderson, Pantaleon, Wlodek, & Moritz, 2015; Gardebjer et al., 2017) and reports of increased rates of obesity in children and adolescents with fetal alcohol spectrum disorders (FASDs) (Fuglestad et al., 2014; Werts, Van Calcar, Wargowski, & Smith, 2014). Structural and functional abnormalities of the central nervous system are common in FASDs (Astley, 2010) and could potentially underpin other phenotypes associated with prenatal ethanol exposure.

In this study, we examined the consequences of prenatal ethanol exposure on brain structure at adolescence and adulthood using ultra-high field magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI). Long-term effects on energy metabolism, body weight and body composition were also explored. Notably, ethanol exposure was restricted to early gestation — equivalent to the period between conception and pregnancy recognition in humans — when the chances of alcohol consumption by pregnant women are high (McCormack et al., 2017), but information on possible adverse outcomes is limited.

## Materials and Methods

### *Prenatal ethanol exposure*

Animal work was conducted in accordance with the Australian code for the care and use of animals for scientific purposes, and was approved by Animal Ethics Committees at the Queensland Institute of Medical Research (P986, A0606-609M) and The University of Queensland (MRI-UQ/TRI/430/13). Prenatal ethanol exposure involved consumption of 10% (v/v) ethanol by pregnant C57BL/6J dams from 0.5 to 8.5 days post coitum (dpc) and was performed as described previously (Kaminen-Ahola, Ahola, Maga, et al., 2010). C57BL/6J mice show a significant preference for 10% (v/v) ethanol when given *ad libitum* two-bottle choice between ethanol and water (Belknap, Crabbe, & Young, 1993). In this study, single-bottle administration of 10% (v/v) ethanol was used to reduce variation in ethanol exposure and, consequently, the number of animals needed for the experiment. This approach is potentially more stressful than a two-bottle choice paradigm. Animal welfare during the exposure period was monitored by measurements of fluid intake, body weight and daily observations of mouse appearance and behaviour. Adult (6-8 week old) C57BL/6J mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia) and acclimatized to a 12-hour light/dark cycle for up to one week. Males were then caged with a single female overnight and detection of a vaginal plug the next morning indicated that mating had taken

place (designated 0.5 dpc). Males were then removed and females were provided with a drink bottle containing either 10% (v/v) ethanol (ethanol-exposed) or water (control) *ad libitum* for the following eight days. Liquid consumption (to the nearest 0.2 ml) was measured every 24 hours. At 8.5 dpc, the ethanol-exposed females were placed back on water. All mice had free access to standard mouse chow (Irradiated Rat and Mouse Diet, Specialty Feeds, Glen Forrest, WA, Australia) at all times. Body weight was measured at 0.5 dpc and 8.5 dpc for ethanol-exposed and control dams, and at 12 weeks of age for their offspring.

### ***MRI and DTI studies***

At P28 or P64, male mice were transcardially perfused with 4% paraformaldehyde according to standard protocols. The brains were then removed and scanned *ex vivo* as described previously (Kurniawan et al., 2014). Brain samples were incubated in 0.2% (v/v) Magnevist (Bayer AG, Leverkusen, Germany) for 4 days prior to imaging, and MRI scans were performed using a Bruker 16.4 Tesla widebore Avance II NMR spectrometer (Bruker Biospin, Karlsruhe, Germany) and 15 mm SAW coil (M2M Imaging, Brisbane, Australia). Whole brain scans were performed using: (1) 3D diffusion-weighted spin-echo sequence at 100  $\mu\text{m}$  isotropic resolution, with 30 diffusion directions,  $b=5000 \text{ s/mm}^2$ ; and (2)  $T_1/T_2^*$ -weighted 3D gradient echo sequence at 50  $\mu\text{m}$  isotropic resolution. The total acquisition time was ~16 hours. Using the C57BL/6 MRI brain atlas (Ma et al., 2005), regions of interest (ROI) were registered to each gradient echo dataset using FSL linear (FLIRT) and non-linear (FNIRT) registration protocols ([www.fmrib.ox.ac.uk/fsl](http://www.fmrib.ox.ac.uk/fsl)). Registered ROIs were then examined and manually corrected using a histology-based C57BL/6 mouse brain atlas (Franklin & Paxinos, 2008). Volumes were calculated using ITK-SNAP (Yushkevich et al., 2006). DiffusionToolkit (Ruopeng Wang, Van J. Wedeen, TrackVis.org, Martinos Center for Biomedical Imaging, Massachusetts General Hospital) was used to process the diffusion data and calculate the DTI parametric maps. Fibretracking was performed using Q-ball and fibre

assignment for continuous tract (FACT). Reconstructed DTI data was visualized and analysed using TrackVis software (Ruopeng Wang, Van J. Wedeen, TrackVis.org, Martinos Center for Biomedical Imaging, Massachusetts General Hospital). All MRI and DTI analyses were conducted blind to treatment group.

### ***Dual-energy X-ray absorptiometry (DXA)***

Body composition was analysed using a PIXImus2 densitometer and software version 2.10 (Lunar, Madison, WI, USA). The head was excluded from all analyses. Measurements automatically supplied by the software included lean tissue (g), fat tissue (g), % fat, bone mineral content (g), bone area (cm<sup>2</sup>) and bone mineral density (g/cm<sup>2</sup>).

### ***Indirect calorimetry***

PhenoMaster metabolic cages (TSE Systems, Chesterfield, MO, USA) were used to monitor food (g) and water (ml) intake, body weight (g), oxygen consumption (VO<sub>2</sub>; ml/h/kg) and carbon dioxide production (VCO<sub>2</sub>; ml/h/kg) in a home cage environment. Mice were housed individually at 22 °C on a 12-hour light/12-hour dark cycle, with paper bedding and free access to water and standard chow (Irradiated Rat and Mouse Diet, Specialty Feeds, Glen Forrest, WA, Australia). Data was collected at 20 minute intervals for 3 days following an initial acclimatization period of 2-3 days. Respiratory exchange ratio (RER) was calculated as the ratio between VCO<sub>2</sub> and VO<sub>2</sub>. Daily energy expenditure was calculated as described by Meyer and colleagues (Meyer, Reitmeir, & Tschop, 2015) using the Heldmaier conversion equation.

### ***Statistics***

Previous work with this model has shown that early gestational ethanol exposure affects offspring outcomes in a stochastic manner, producing substantial intra-litter variation even in inbred mice (Kaminen-Ahola, Ahola, Maga, et al., 2010; Zhang, Ho, Vega, Burne, & Chong, 2015). Therefore each animal was considered an independent unit of analysis (Elswick,



Welsch, & Janszen, 2000). Statistical analyses were conducted using either *R* (R Development Core Team, 2010) or GraphPad Prism 6. The Student's *t*-test was used to analyse differences between treatment groups (ethanol-exposed and control) for maternal liquid consumption and weight gain over the 8-day exposure period, litter size at weaning and offspring brain structure. Adjustment for multiple testing used the Holm-Sidak method. Two-way ANOVAs with a Tukey's multiple comparison *post hoc* test were used to analyse the effects of sex, treatment and the interaction of sex and treatment on offspring body weight, body composition, food and water intake and energy metabolism.

## Results

### *Consumption of 10% (v/v) ethanol by C57BL/6J female mice*

As in our previous studies (Kaminen-Ahola, Ahola, Maga, et al., 2010; Zhang et al., 2015), there was no effect of ethanol treatment on the average volume of liquid consumed per day by pregnant dams, maternal body weight gain during the exposure period (0.5 to 8.5 dpc) or litter size at weaning (Figure 1), indicating that the exposure was not detrimental to maternal health or offspring viability.

### *Structural MRI in adolescent and adult male offspring exposed to ethanol in utero*

The volumes of the whole brain, neocortex, cerebellum, caudate putamen, hippocampus and hypothalamus were compared between ethanol-exposed and control male offspring both at adolescence (P28) and adulthood (P64). There was no effect of prenatal ethanol exposure on whole brain volume at either age (P28: control =  $366.30 \pm 7.48 \text{ mm}^3$ , ethanol-exposed =  $344.92 \pm 11.07 \text{ mm}^3$ , unadjusted  $P=0.15$ ; P64: control =  $369.46 \pm 5.69 \text{ mm}^3$ , ethanol-exposed =  $370.32 \pm 4.70 \text{ mm}^3$ , unadjusted  $P=0.91$ ). At P28, all of the other brain regions that were analysed were similarly unaffected by ethanol exposure (Table 1). At P64, there was a trend towards a smaller hippocampus (3.8%, adjusted  $P=0.053$ ) and larger hypothalamus (4.3%, adjusted  $P=0.17$ ) in ethanol-exposed offspring, but the differences were not statistically

significant (Table 1). Volumetric analysis of hippocampal subfields in adult males, including the dentate gyrus and cornu ammonis (CA) 1-2 and 3 regions, found no significant differences between treatment groups, suggesting that the reduction in overall hippocampal volume was not caused by a change in any one specific subregion (Supplementary Figure S1 and Table 2).

***DTI analysis of white matter microstructure in adolescent and adult male offspring exposed to ethanol in utero***

The anterior commissure, corpus callosum, hippocampal commissure, internal capsule, optic tract, and thalamus were analysed by DTI. Comparisons were made between treatment groups for fractional anisotropy as well as fibre tract number, volume and length. At P28 a reduction in fibre tract length was observed in the hippocampal commissure of ethanol-exposed mice (unadjusted  $P < 0.05$ ) however this resolved by adulthood (Tables 3 and 4). None of the other regions analysed were altered at either P28 or P64 indicating that white matter integrity and connectivity were not substantially affected by ethanol exposure early in pregnancy.

***Ethanol exposure early in pregnancy is associated with changes in energy metabolism and body composition in adulthood***

Body weight and body composition were measured in both male and female offspring of ethanol-exposed and control dams at 12 weeks of age (Figure 2 and Figure 3). Energy intake and expenditure were measured in littermates at 21-26 weeks of age (Figure 4 and Figure 5). There was an effect of sex on body weight ( $F(1,92)=547.8$ ,  $P < 0.0001$ ), lean mass ( $F(1,49)=196.2$ ,  $P < 0.0001$ ) and fat mass ( $F(1,49)=23.3$ ,  $P < 0.0001$ ), with adult male offspring being significantly heavier than female offspring in the same treatment group (Figure 2a-c). Ethanol exposure had no significant effect on body weight or lean mass in either sex (Figure 2a-b), but ethanol-exposed males exhibited increased (18.7%; control = 2.9

$\pm 0.5$  g, ethanol-exposed =  $3.5 \pm 0.4$  g) fat mass compared to control males (treatment  $F(1,49)=4.7$ ,  $P<0.05$ ; treatment x sex interaction  $F(1,49)=5.1$ ,  $P<0.05$ , Figure 2c). Furthermore, the increased adiposity in ethanol-exposed males resulted in percentage fat levels (~14%) similar to that of female offspring (Figure 2d). Prenatal ethanol exposure had no effect on bone mineral content, bone area or bone mineral density in either sex, although there was a significant effect of sex on bone mineral content and bone area (Figure 3). Metabolic phenotyping by indirect calorimetry revealed significant differences in oxygen consumption ( $VO_2$ ; sex  $F(1,37)=18.9$ ,  $P<0.001$ , treatment  $F(1,37)=9.6$ ,  $P<0.01$ ), carbon dioxide production ( $VCO_2$ ; sex  $F(1,37)=12.9$ ,  $P<0.001$ , treatment  $F(1,37)=6.4$ ,  $P<0.05$ ) and daily energy expenditure (sex  $F(1,37)=11.08$ ,  $P<0.01$ , treatment  $F(1,37)=4.9$ ,  $P<0.05$ ) by sex and by treatment group (Figure 4a, b and d, respectively). Moreover, ethanol-exposed males tended to have lower oxygen consumption (9.1%, adjusted  $P=0.10$ ), carbon dioxide production (8.8%, adjusted  $P=0.16$ ) and daily energy expenditure (7.9%, adjusted  $P=0.11$ ) compared to sex- and age-matched controls (Figure 4a, b and d, respectively). There was no effect of sex or treatment on offspring respiratory exchange ratio (RER, Figure 4c). In addition, ethanol exposure did not significantly affect food or water intake in offspring of either sex (Figure 5) although there was an effect of light cycle on these measures (male water intake:  $F(1,34)=410.9$ ,  $P<0.0001$ ; female water intake:  $F(1,40)=98.7$ ,  $P<0.0001$ ; male food intake:  $F(1,34)=391.7$ ,  $P<0.0001$ ; female food intake:  $F(1,40)=131.0$ ,  $P<0.0001$ ).

## Discussion

Recognition of pregnancy by women often results in the reduction of risky behaviours such as alcohol consumption; however, this does not usually occur before the fourth week of gestation. Thus, there is a critical window of early development when inadvertent alcohol exposure is possible, warranting investigation of its impact on offspring health. We have an established inbred C57BL/6J mouse model of gestational ethanol exposure which

encompasses implantation, gastrulation and early organogenesis, and is developmentally equivalent to the first 3-4 weeks of a human pregnancy (Kaminen-Ahola, Ahola, Maga, et al., 2010). Prior work has shown that this type of exposure is capable of producing changes in adolescent body weight (Kaminen-Ahola, Ahola, Flatscher-Bader, et al., 2010; Kaminen-Ahola, Ahola, Maga, et al., 2010) and craniofacial structure (Kaminen-Ahola, Ahola, Maga, et al., 2010) as well as adult behaviour (Sanchez Vega, Chong, & Burne, 2013). In this report, we extend our studies to show that early gestational ethanol exposure can also have long-term consequences on offspring brain structure, energy metabolism and body composition.

This study has several limitations. First, the blood alcohol concentrations in female mice consuming the ethanol are unknown. Second, imaging at P28 involved a small number of samples and was likely underpowered to detect subtle changes in brain structure. Third, MRI and DTI were not performed on female offspring. Therefore it remains unclear whether early gestational ethanol exposure affects brain structure in females in a similar manner to that observed in males.

Previous brain imaging studies in mouse models of prenatal ethanol exposure have focused on the impact of acute high dose exposures (blood alcohol concentrations of 350–420 mg/dl) and have shown alterations in both grey and white matter, although the negative effect of these exposures on offspring viability has largely prevented the examination of long-term consequences (O'Leary-Moore, Parnell, Lipinski, & Sulik, 2011). High dose ethanol exposures on gestational day (GD) 7, 8 or 10 affected overall brain volume, the forebrain, lateral ventricles, olfactory bulbs, hippocampus and cerebellum in fetuses at GD17 (Godin et al., 2009; O'Leary-Moore et al., 2010; Parnell et al., 2009). Furthermore, different structures were affected depending on the day of the exposure (O'Leary-Moore et al., 2011). A DTI study following high dose ethanol exposure on GD7 revealed effects on the fetal internal and external capsule, fimbria/fornix and corpus callosum at GD17 (O'Leary-Moore et al., 2011).

Cao and colleagues used quantitative susceptibility mapping to identify abnormalities in the anterior commissure, hippocampal commissure and corpus callosum in mice at P45 (equivalent to 10–12 years in humans) after high dose exposure at GD7; however, analysis of the same midline structures using DTI failed to identify any differences between the ethanol-exposed and control mice (Cao et al., 2014).

Maternal consumption of 10% (v/v) ethanol likely results in lower blood alcohol concentrations than two intraperitoneal injections (4 hours apart) of 23–25% (v/v) ethanol at 2.8–2.9 g/kg, as used in the acute high dose studies, and is expected to be closer to a moderate exposure (Allan, Chynoweth, Tyler, & Caldwell, 2003). In contrast to studies using acute high dose exposures, we found that most grey and white matter structures were either consistently unaffected by early gestational ethanol exposure, or exhibited transient changes in adolescence which were resolved by adulthood. At P64 (9 weeks of age), when brain development is complete, we identified disproportionate volumetric changes in both the hippocampus and the hypothalamus; however, the differences were not statistically significant following correction for multiple comparisons, possibly due to the small magnitude of the changes involved (~4%). The lack of significant white matter changes in adult mice suggests that these structures are not susceptible to the type of exposure used in this study; however, we cannot exclude the possibility that DTI may not be sensitive enough to detect subtle changes (Cao et al., 2014).

The hippocampus is known to be particularly sensitive to intrauterine alcohol exposure (Autti-Ramo et al., 2002). Neuroimaging studies have revealed changes in hippocampal volume, shape and neurometabolites (Moore, Migliorini, Infante, & Riley, 2014; Wang & Kroenke, 2015). Moreover, the type and severity of brain structural changes vary with the timing, dosage and duration of ethanol exposure. Our finding of limited changes in brain structure overall following early gestational ethanol exposure supports the idea that the

hippocampus and hypothalamus may be more vulnerable to ethanol-induced damage. Altered hippocampal structure in individuals with FASD has been correlated with performance in learning and memory tests (Coles et al., 2011; Willoughby, Sheard, Nash, & Rovet, 2008). Behavioural profiling of adult mice subjected to the same type of ethanol exposure as used in this study identified alterations in performance in the Morris water maze (Sanchez Vega et al., 2013); however, further work is necessary to determine the extent to which the changes in hippocampal structure influence learning and memory in this model.

Changes in hypothalamic structure have previously been documented in humans and animals following binge-like prenatal ethanol exposure (Coulter, Leech, Schaefer, Scheithauer, & Brumback, 1993; Fish et al., 2016). Our results show that ethanol exposure restricted to early pregnancy is sufficient to influence the volume of the hypothalamus in adult male offspring. The basis of this hypothalamic enlargement is unknown, but could involve increased neurogenesis similar to that reported in rats after low-moderate ethanol exposure in mid-late pregnancy (GD9-21) (Chang, Karatayev, Liang, Barson, & Leibowitz, 2012). Furthermore, deficits in hypothalamic function have been reported in rats after low-moderate ethanol exposures either late in gestation (Abate, Hernandez-Fonseca, Reyes-Guzman, Barbosa-Luna, & Mendez, 2014) or throughout gestation (Dembele, Yao, Chen, & Nyomba, 2006; Glavas, Ellis, Yu, & Weinberg, 2007).

The hypothalamus is an important regulator of metabolism and energy homeostasis and structural damage in this region has been linked with long-term weight gain and obesity in humans (Pinkney, Wilding, Williams, & MacFarlane, 2002). We found a significant increase in adiposity (18.7% or an average of 0.6 g) specifically in adult male offspring as a consequence of early pregnancy ethanol exposure; however, this did not translate into a substantial change in body weight, possibly due to a subtle (0.3 g) but non-significant decrease in lean tissue in the same group. A recent study using a similar, periconceptional,

ethanol exposure combined with a postnatal high-fat diet in rats reported similar, male-specific, effects on fat mass, fat-free mass and body weight (Gardebjer et al., 2017). It remains unclear whether the change in fat mass represents a uniform increase across all fat depots, or whether some depots are affected to a greater extent than others. Metabolic phenotyping revealed reduced energy expenditure, but no change in energy (food) intake, in adult ethanol-exposed offspring of both sexes, but tending to be stronger in males. The decrease in energy expenditure could be due to primary perturbations in basal metabolic rate, thermoregulation and/or physical activity; however, further work is required to determine whether one or all of these factors are involved. Further work is also necessary to discern whether the increased adiposity in ethanol-exposed males is a cause or consequence of their altered energy balance.

There is increasing evidence for sex-specific responses to a variety of adverse environmental exposures *in utero* (Bolton, Auten, & Bilbo, 2014; Giesbrecht, Letourneau, Campbell, Alberta Pregnancy, & Nutrition Study, 2016; Paolozza, Munn, Munoz, & Reynolds, 2015) as well as for intrinsic sex differences in energy metabolism and the development of obesity (Fried, Lee, & Karastergiou, 2015; Mauvais-Jarvis, 2015). In this study, early gestational ethanol exposure significantly affected fat mass specifically in adult male offspring. The mechanisms underpinning this difference between male and female offspring are not known, but could involve sex hormones and/or sexually dimorphic gene expression. We have previously identified male-specific changes in both gene expression and epigenetic state in the adult hippocampus following early gestational ethanol exposure (Zhang et al., 2015), lending support to the idea that similar modifications could occur in tissues (e.g. hypothalamus, adipose tissue) relevant to the phenotype described here.

In summary, our ultra-high field MRI study indicates that ethanol exposure early in pregnancy has limited effects on long-term brain structure in male mice, with only subtle

changes in the hippocampus and hypothalamus. Our results are also consistent with the idea that ethanol-induced changes in hypothalamic structure contribute to perturbations in energy metabolism and altered body composition in later life.

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**Figure Legends**

**Figure 1.** Maternal liquid consumption per day **(a)** and % weight gain **(b)** over the 8-day ethanol exposure period (0.5-8.5 dpc). **(c)** Litter size at weaning (P21). EtOH: ethanol-exposed mice. All data points are overlaid on box and whisker graphs where the box extends from the 25th to 75th percentile, the line in the middle is the median and the whiskers show the maximum and minimum values obtained.

**Figure 2.** Body weight and body composition of adult (12 week old) male and female offspring. Control offspring are indicated by open circles ( $\circ$ ) and ethanol-exposed (EtOH) offspring are indicated by filled circles ( $\bullet$ ). **(a)** Body weight of control males (n=21 from 6 litters), EtOH males (n=23 from 7 litters), control females (n=23 from 6 litters) and EtOH females (n=29 from 7 litters). Lean mass **(b)**, fat mass **(c)** and % fat **(d)** are also shown for a subset of control males (n=11 from 6 litters), EtOH males (n=14 from 7 litters), control females (n=12 from 6 litters) and EtOH females (n=16 from 7 litters). All data points are overlaid on box and whisker graphs where the box extends from the 25th to 75th percentile, the line in the middle is the median and the whiskers show the maximum and minimum values obtained; Tukey's multiple comparisons test  $*P<0.05$ ,  $****P<0.0001$ .

**Figure 3.** Bone mineral content (BMC), bone area and bone mineral density (BMD) of adult (12 week old) male and female offspring. Control offspring are indicated by open circles ( $\circ$ ) and ethanol-exposed (EtOH) offspring are indicated by filled circles ( $\bullet$ ). BMC **(a)**, bone area **(b)** and BMD **(c)** are plotted for four groups of mice consisting of control males (n=11 from 6 litters), EtOH males (n=14 from 7 litters), control females (n=12 from 6 litters) and EtOH females (n=16 from 7 litters). All data points are overlaid on box and whisker graphs where the box extends from the 25th to 75th percentile, the line in the middle is the median and the whiskers show the maximum and minimum values obtained; Tukey's multiple comparisons test  $***P<0.001$ ,  $****P<0.0001$ .

**Figure 4.** Energy metabolism in adult (21-26 week old) male and female offspring. Control offspring are indicated by open circles ( $\circ$ ) and ethanol-exposed (EtOH) offspring are indicated by filled circles ( $\bullet$ ). **(a)** Oxygen consumption ( $\text{VO}_2$ ), **(b)** carbon dioxide production ( $\text{VCO}_2$ ), **(c)** respiratory exchange ratio (RER) and **(d)** daily energy expenditure. Control male (n=10 from 6 litters), EtOH male (n=9 from 7 litters), control female (n=10 from 6 litters) and EtOH female (n=12 from 7 litters) data points are overlaid on box and whisker graphs where the box extends from the 25th to 75th percentile, the line in the middle is the median and the whiskers show the maximum and minimum values obtained; Tukey's multiple comparisons test \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

**Figure 5.** Water and food intake by adult (21-26 week old) male and female offspring. Control offspring are indicated by open circles ( $\circ$ ) and ethanol-exposed (EtOH) offspring are indicated by filled circles ( $\bullet$ ). **(a-b)** water intake and **(c-d)** food intake were analysed separately for the light and dark phases of the light cycle. Control male (n=10 from 6 litters), EtOH male (n=9 from 7 litters), control female (n=10 from 6 litters) and EtOH female (n=12 from 7 litters) data points are overlaid on box and whisker graphs where the box extends from the 25th to 75th percentile, the line in the middle is the median and the whiskers show the maximum and minimum values obtained; Tukey's multiple comparisons test \*\*\*\* $P < 0.0001$ .



**Table 2.** Volumetric analysis of hippocampal subfields in male offspring at P64 (n=11 per group from 9 litters)

Hippocampal subfield	Relative Volume <sup>a</sup>		<i>P</i> -value
	Control	Ethanol-exposed	
Dentate gyrus	0.0119 ± 0.00121	0.0116 ± 0.000506	0.47
CA1 and CA2	0.0265 ± 0.00206	0.0265 ± 0.00159	1.00
CA3	0.00362 ± 0.00118	0.00382 ± 0.000929	0.67

<sup>a</sup>Mean±SD. All volumes were normalised to whole brain volume.

**Table 3.** DTI analysis at P28 (n=5 per group from 2-3 litters)

Region	Parameter	Mean $\pm$ SD		P-value
		Control	Ethanol-exposed	
AC	FA	0.36 $\pm$ 0.06	0.37 $\pm$ 0.01	0.55
	FT number	762 $\pm$ 305	547 $\pm$ 222	0.24
	FT Volume <sup>a</sup>	3 109 $\pm$ 1 056	2 258 $\pm$ 679	0.17
	FT Length (mm)	7.99 $\pm$ 1.98	6.57 $\pm$ 1.72	0.26
CC	FA	0.36 $\pm$ 0.03	0.35 $\pm$ 0.03	0.40
	FT number	6 920 $\pm$ 1 829	6 197 $\pm$ 2 823	0.65
	FT Volume <sup>a</sup>	19 663 $\pm$ 4 261	18 341 $\pm$ 6 902	0.73
	FT Length (mm)	3.77 $\pm$ 0.88	3.64 $\pm$ 1.04	0.84
HC	FA	0.42 $\pm$ 0.03	0.40 $\pm$ 0.03	0.29
	FT number	3 293 $\pm$ 548	2 777 $\pm$ 731	0.25
	FT Volume <sup>a</sup>	8 776 $\pm$ 2 235	7 236 $\pm$ 1 588	0.25
	FT Length (mm)	5.00 $\pm$ 0.32	4.50 $\pm$ 0.30	0.03*
IC	FA	0.30 $\pm$ 0.01	0.30 $\pm$ 0.01	1.00
	FT number	5 825 $\pm$ 518	4 562 $\pm$ 1 035	0.05
	FT Volume <sup>a</sup>	14 522 $\pm$ 1 235	12 164 $\pm$ 2 357	0.09
	FT Length (mm)	5.22 $\pm$ 0.37	4.76 $\pm$ 0.30	0.07
OT	FA	0.44 $\pm$ 0.02	0.42 $\pm$ 0.02	0.29
	FT number	429 $\pm$ 105	415 $\pm$ 132	0.37
	FT Volume <sup>a</sup>	1 588 $\pm$ 153	1 421 $\pm$ 267	0.74
	FT Length (mm)	3.62 $\pm$ 0.23	3.71 $\pm$ 0.41	0.78
TH	FA	0.21 $\pm$ 0.02	0.19 $\pm$ 0.02	0.97
	FT number	1 251 $\pm$ 324	1 128 $\pm$ 362	0.54
	FT Volume <sup>a</sup>	3 148 $\pm$ 681	2 989 $\pm$ 813	0.87
	FT Length (mm)	1.48 $\pm$ 0.23	1.34 $\pm$ 0.16	0.82

SD: standard deviation, AC: anterior commissure, CC: corpus callosum, HC: hippocampal commissure, IC: internal capsule, OT: optic tract, TH: thalamus, FA: fractional anisotropy and FT: fibre tract. <sup>a</sup>Volume is shown as voxel numbers. Each voxel is 10<sup>-6</sup> mm<sup>3</sup>. \*P<0.05.



**Table 4.** DTI analysis at P64 (n=11 per group from 9 litters)

Region	Parameter	Mean $\pm$ SD		P-value
		Control	Ethanol-exposed	
AC	FA	0.45 $\pm$ 0.05	0.45 $\pm$ 0.04	0.79
	FT number	457 $\pm$ 83	534 $\pm$ 160	0.38
	FT Volume <sup>a</sup>	2 334 $\pm$ 331	2 600 $\pm$ 839	0.54
	FT Length (mm)	7.89 $\pm$ 0.76	6.25 $\pm$ 1.45	0.07
CC	FA	0.43 $\pm$ 0.01	0.44 $\pm$ 0.01	0.62
	FT number	7 008 $\pm$ 612	6 919 $\pm$ 621	0.82
	FT Volume <sup>a</sup>	19 904 $\pm$ 1 469	19 454 $\pm$ 1 801	0.68
	FT Length (mm)	6.23 $\pm$ 0.81	5.57 $\pm$ 0.28	0.15
HC	FA	0.42 $\pm$ 0.01	0.42 $\pm$ 0.02	0.73
	FT number	4 613 $\pm$ 884	4 447 $\pm$ 848	0.77
	FT Volume <sup>a</sup>	10 702 $\pm$ 1 733	10 562 $\pm$ 2 153	0.91
	FT Length (mm)	5.96 $\pm$ 0.51	5.93 $\pm$ 0.55	0.93
IC	FA	0.32 $\pm$ 0.02	0.32 $\pm$ 0.02	0.89
	FT number	3 157 $\pm$ 888	2 550 $\pm$ 741	0.28
	FT Volume <sup>a</sup>	8 625 $\pm$ 2 049	7 438 $\pm$ 1 350	0.32
	FT Length (mm)	4.31 $\pm$ 0.43	4.60 $\pm$ 0.54	0.38
OT	FA	0.45 $\pm$ 0.02	0.44 $\pm$ 0.02	0.33
	FT number	498 $\pm$ 109	428 $\pm$ 134	0.39
	FT Volume <sup>a</sup>	1 944 $\pm$ 211	1 864 $\pm$ 327	0.66
	FT Length (mm)	3.68 $\pm$ 0.33	4.08 $\pm$ 0.57	0.22
TH	FA	0.20 $\pm$ 0.02	0.19 $\pm$ 0.03	0.65
	FT number	1 354 $\pm$ 378	1 136 $\pm$ 388	0.39
	FT Volume <sup>a</sup>	4 449 $\pm$ 789	3 789 $\pm$ 960	0.27
	FT Length (mm)	2.88 $\pm$ 0.39	2.54 $\pm$ 0.30	0.17

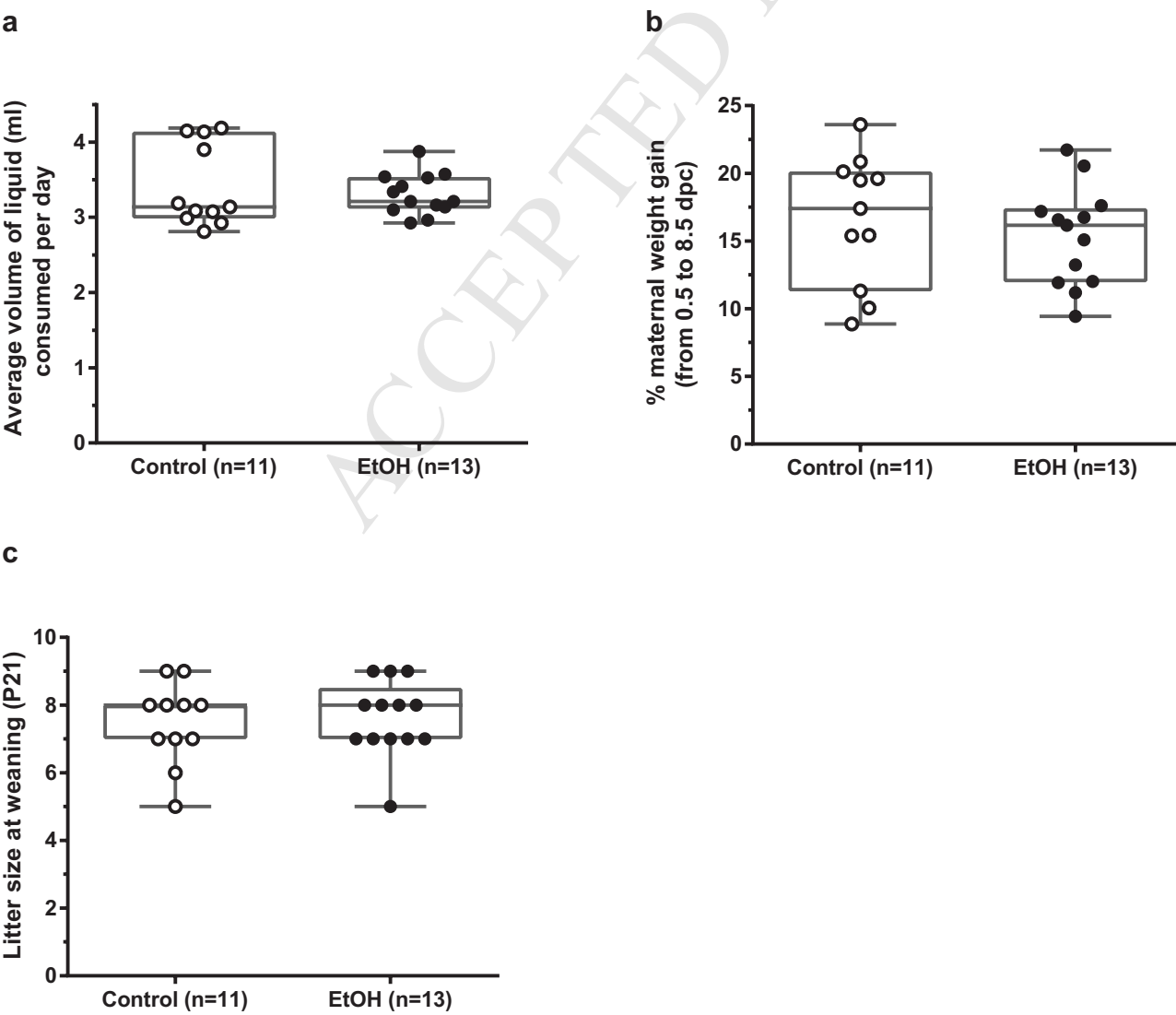
SD: standard deviation, AC: anterior commissure, CC: corpus callosum, HC: hippocampal commissure, IC: internal capsule, OT: optic tract, TH: thalamus, FA: fractional anisotropy and FT: fibre tract. <sup>a</sup>Volume is shown as voxel numbers. Each voxel is 10<sup>-6</sup> mm<sup>3</sup>.

**Table 1.** Relative volumes of selected brain regions in adolescent (P28) and adult (P64) male offspring

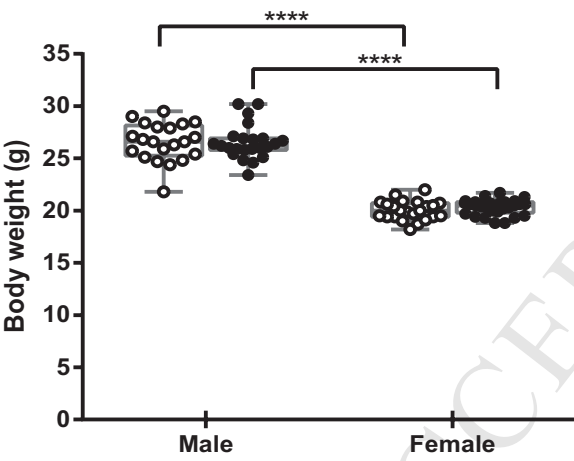
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Brain region	Relative volume <sup>a</sup>		Raw <i>P</i> -value	Adjusted <i>P</i> -value
	Control	Ethanol-exposed		
P28 (n=5/group from 2-3 litters)				
Neocortex	0.343 ± 0.00910	0.342 ± 0.00600	0.79	0.99
Cerebellum	0.111 ± 0.00616	0.109 ± 0.00926	0.71	0.99
Caudate putamen	0.0536 ± 0.000673	0.0535 ± 0.00170	0.90	0.99
Hippocampus	0.0503 ± 0.00160	0.0505 ± 0.00360	0.94	0.99
Hypothalamus	0.0211 ± 0.00163	0.0217 ± 0.00223	0.65	0.99
P64 (n=11/group from 9 litters)				
Neocortex	0.314 ± 0.00563	0.310 ± 0.0108	0.40	0.68
Cerebellum	0.123 ± 0.00559	0.122 ± 0.00393	0.59	0.68
Caudate putamen	0.0534 ± 0.00258	0.0523 ± 0.00257	0.32	0.68
Hippocampus	0.0533 ± 0.00141	0.0515 ± 0.00159	0.011*	0.053
Hypothalamus	0.0228 ± 0.000830	0.0235 ± 0.000808	0.045*	0.17

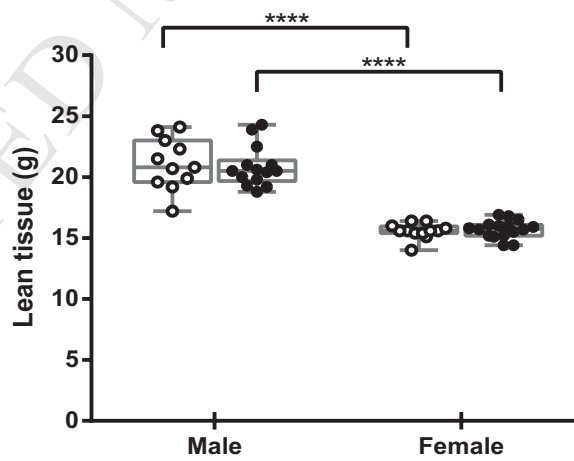
<sup>a</sup>Mean±SD. All volumes were normalized to whole brain volume to minimize the effects of subtle inter-individual variation. \**P*<0.05



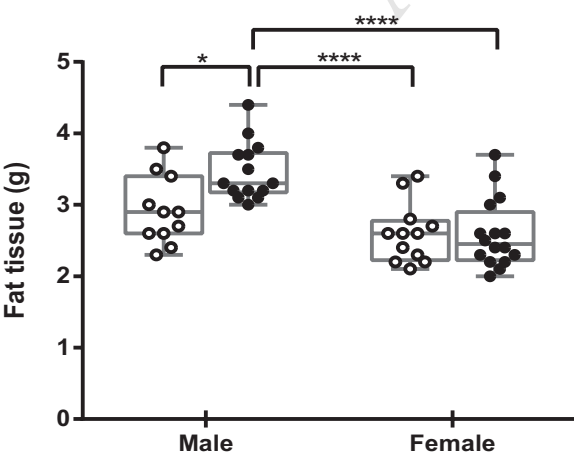
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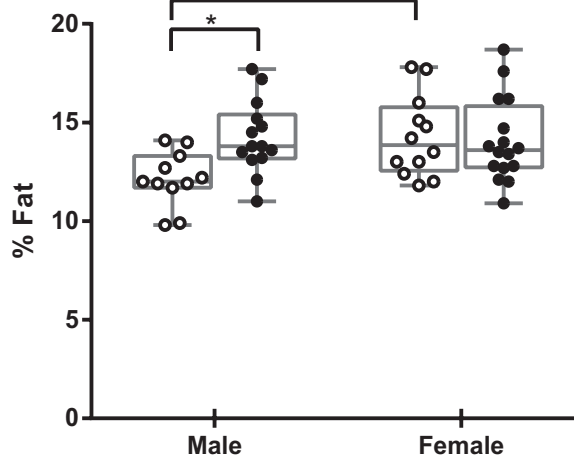
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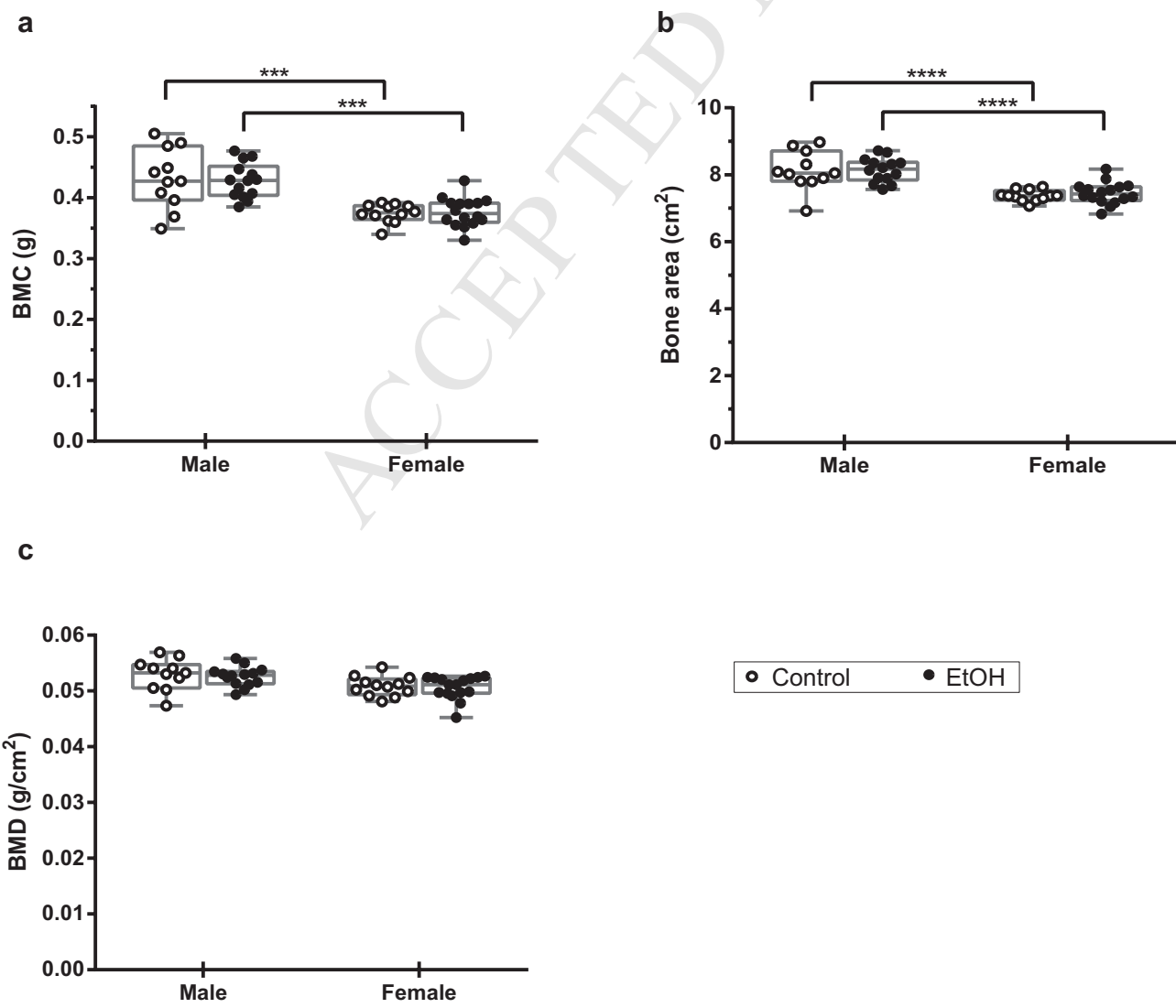


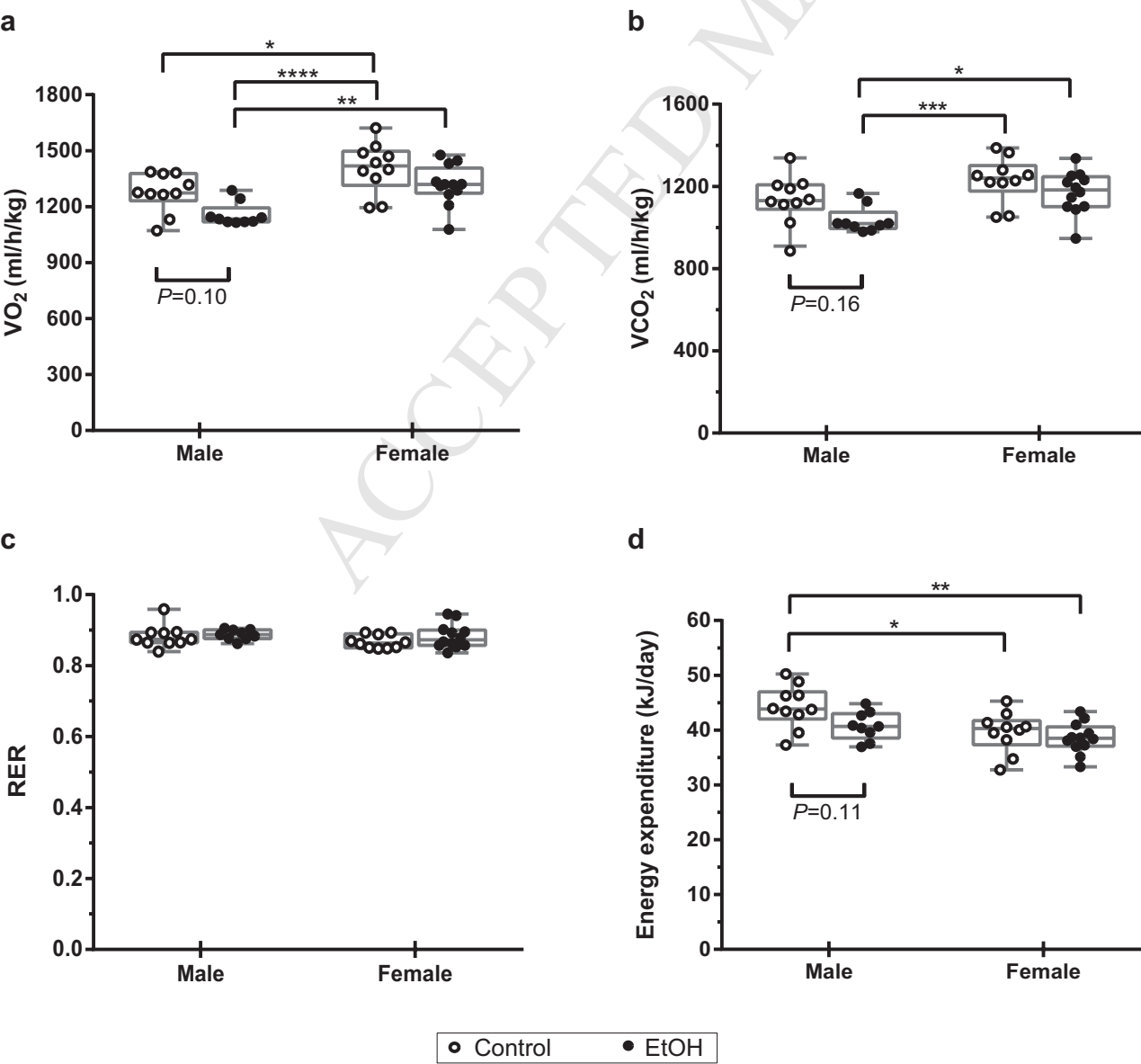
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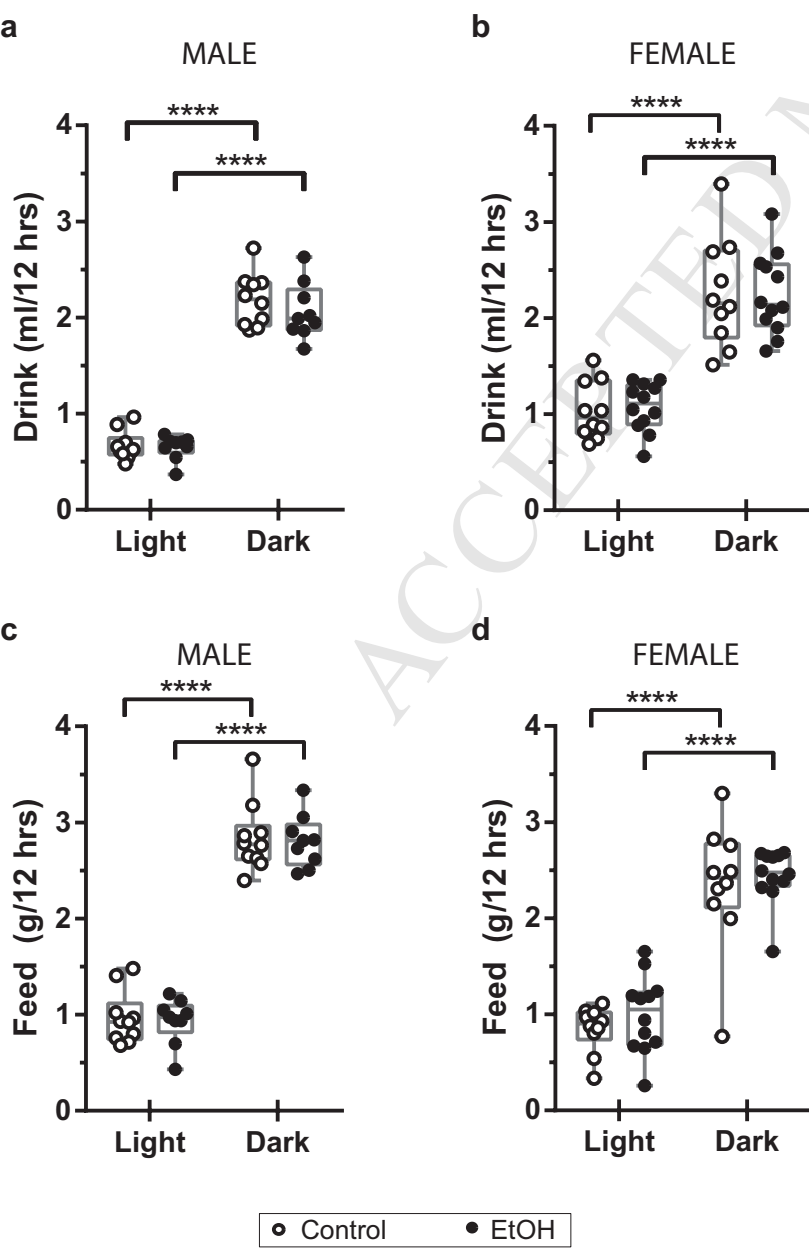


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## 1 **Highlights**

- 2       • The long-term effects of early pregnancy alcohol exposure were examined in mice.
- 3       • Hippocampal and hypothalamic structure were altered in adult male offspring.
- 4       • Energy metabolism and body composition were also changed in adult males.
- 5       • CNS abnormalities may underpin other outcomes linked to prenatal alcohol exposure.